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Intrathecal Adenosine following Spinal Nerve Ligation in Rat

Short Residence Time in Cerebrospinal Fluid and No Change in A_1 Receptor Binding

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Background: **Intrathecal adenosine produces a remarkably prolonged effect to relieve mechanical hypersensitivity after peripheral nerve injury in animals. The purpose of the current study was to investigate whether this reflected an alteration in kinetics of adenosine in cerebrospinal fluid or in the number of spinal A_1 adenosine receptors after nerve injury.**

Methods: **Male rats were anesthetized, and the left L5 and L6 spinal nerves were ligated. Two weeks later, a lumbar intrathecal catheter and intrathecal space microdialysis catheter were inserted. Adenosine, 20 μg, was injected intrathecally in these and in normal rats, and microdialysates of the intrathecal space were obtained. Radioligand binding studies of adenosine A_1 receptors were determined in spinal cord tissue from other normal and spinal nerve–ligated rats.**

Results: **Adenosine disappeared from rat cerebrospinal fluid within 30 min after intrathecal injection, with no difference between normal and spinal nerve–ligated animals. A_1 adenosine receptor binding sites in the spinal cord were increased after spinal nerve ligation. This increase disappeared when adenosine deaminase was added to the membrane homogenates, suggestive of decreased endogenous adenosine in the membranes of nerve-ligated animals.**

Conclusion: **These data show that prolonged alleviation of hypersensitivity observed with intrathecal adenosine in this animal model of neuropathic pain is not due to prolonged residence in cerebrospinal fluid, although pharmacokinetics in tissues are unknown. Similarly, there is no evidence for upregulation in spinal A_1 adenosine receptors after spinal nerve ligation, and the adenosine deaminase experiment is consistent with a depletion of adenosine in spinal cord tissue after spinal nerve ligation.**

A_1 ADENOSINE receptor stimulation produces analgesia by both peripheral and central mechanisms, and a variety of molecules are being developed, including direct agonists, allosteric modulators, and uptake or metabolism inhibitors to provide analgesia through this nonopioid mechanism.[1] Peripherally, A_1 receptor stimulation inhibits excitation of dorsal root ganglion cells in culture[2] and reduces release of calcitonin gene–related peptide and substance P from afferent terminals in the spinal cord.[3,4] Centrally, A_1 receptors are present on spinal cord neurons,[5] and stimulation of these receptors reduces C fiber–driven responses in dorsal horn neurons[6] and causes antinociception to noxious thermal stimuli.[7] Additionally, intrathecal injection of adenosine agonists reduces mechanical allodynia after peripheral nerve injury in rats,[8] a model of hypersensitivity observed in patients with chronic pain, indicating a role in inhibition of central sensitization.

Previous studies have focused on synthetic analogs of adenosine and only indirectly examine the regulation of endogenous adenosine to modulate pain transmission in normal or pathologic conditions. Adenosine is a ubiquitous, water-soluble nucleoside, proposed to function in the nervous system as an inhibitory neuromodulator.[9] Adenosine's action in the extracellular space is terminated mostly by reuptake *via* equilibrative and concentrative transporters and subsequent intracellular metabolism. This metabolism is dependent on two enzymes, adenosine kinase and adenosine deaminase. Adenosine kinase facilitates a phosphorylation of adenosine to ADP and dominates control of extracellular adenosine concentrations under normal circumstances.[10] The half-life of adenosine in blood is a few seconds, primarily because of rapid cellular uptake.

In contrast to the extremely short-lasting effects of adenosine after intravenous administration, we recently observed a prolonged (24-h) antiallodynic effect of intrathecally injected adenosine in rats with peripheral nerve injury.[11] One purpose of the current study was to determine whether this prolonged duration of action reflected a pharmacokinetic mechanism. To test this, we used a recently described method of microdialysis of the intrathecal space[12] in conscious rats with and without peripheral nerve injury and mechanical hypersensitivity. In addition, we previously noted in rats that intrathecal adenosine, in contrast to synthetic analogs, was essentially inactive against acute thermal pain but was active to reduce the degree of allodynia after nerve injury.[11,13] A secondary purpose of the current study was to determine whether this increased efficacy of intrathecal ad-

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ministration of the endogenous compound adenosine in hypersensitivity states reflected up-regulation of A_1 adenosine receptors in the spinal cord in animals.

Materials and Methods

After Animal Care and Use Committee approval (Wake Forest University School of Medicine, Winston-Salem, NC), male rats (Harlan Sprague-Dawley) weighing 200–250 g at the time of surgery were studied. Animals were housed at 22°C and under a 12 h light–12 h dark cycle, with free access to food and water. Spinal nerve ligation was performed as previously described.[14] During halothane anesthesia, the left L5 and L6 spinal nerves were isolated adjacent to the vertebral column and tightly ligated with 6-0 silk sutures distal to the dorsal root ganglion. After a recovery period of 13 days, left paw tactile hypersensitivity was confirmed by measuring hind paw thresholds to von Frey filaments, using an up–down method previously described.[15] Only rats with a threshold below 4 g were further included for study.

In Vivo *Microdialysis*

Sixteen male rats were randomly and equally divided for spinal nerve ligation or no surgery. Thirteen days later, all animals underwent implantation of intrathecal injection and loop dialysis catheters as previously described.[12,16] During halothane anesthesia, polyethylene tubing and a loop microdialysis catheter were inserted through a small hole in the cisterna magna and directed caudad 8 cm such that their tips were in the lumbar intrathecal space. Rats showing neurologic deficits were immediately killed using an overdose of pentobarbital; the other animals were allowed to recover 4 or 5 days before the microdialysis experiment.

On the day of the study, microdialysis catheters were connected to 5-ml syringes containing artificial cerebrospinal fluid (CSF; 151.1 mM Na^+, 2.6 mM K^+, 0.9 mM Mg^{2+}, 1.3 mM Ca^{2+}, 122.7 mM Cl^-, 21 mM HCO_3, 2.5 mM HPO_4, and 3.5 mM dextrose) using a microinfusion pump at a flow rate of 5 μl/min. After a 1-h washout period and two 5-min baseline samples, 20 μg adenosine, in a volume of 10 μl, was injected through the intrathecal catheter, followed by 10 μl saline, and dialysis samples were collected on ice every 5 min for 25 min. Sample analysis was performed using high-pressure liquid chromatography, using a Rainin A_1 autosampler onto a 250 × 4.6 Luna C18 column (Phenomenex, Torrance, CA) at a flow rate of 1.3 ml/min with a mobile phase consisting of 10 mM ammonium phosphate, pH 6.0, with 14% methanol. Adenosine was determined using a Rainin Dynamax Model UV-D II absorbance detector at 254 nm, with limit of sensitivity of 10 pmol per injection and a coefficient of variation at 50 pmol of 7%.

Radioligand Binding

Preliminary experiments were performed to optimize binding conditions for pH, buffer components, adenosine deaminase concentration, time, temperature, and protein concentration. Spinal cords from normal and spinal nerve–ligated rats were quickly harvested and cut into lumbar and thoracic sections. These sections were dissected into left and right, dorsal and ventral quadrants and were stored at −70°C until used. Cords from 9 or 10 animals per group were prepared together. All tissue was stored for 1–4 weeks.

Tissue was thawed, quickly chopped, and then suspended in 170 mM Tris buffer of pH 7.4. Tissue was homogenized and then disrupted by sonication (5–7 s). The suspension was centrifuged at 3,000 rpm for 15 min (4°C). The supernatant was then centrifuged at 23,000 rpm for 30–40 min (4°C). The pellet was resuspended in the same buffer and disrupted by sonication for 5 s. Radioligand binding saturation experiments were performed with 250-μl aliquots of tissue (final volume 1 ml) and increasing concentrations of [^{3}H]-DPCPX (90 pM–7 nM). Nonspecific binding was defined with the presence of 100 μM cold (unlabeled) adenosine. In some experiments, adenosine deaminase (1 IU/ml) was added. Competition experiments were performed with 200-μl aliquots of tissue. The final volume was 0.5 ml, the concentration of [^{3}H]-DPCPX was 500 pM, and 1 IU/ml adenosine deaminase was added to the vials for total binding. The following adenosine A_1 receptor agonists were used in increasing concentrations: 2-chloroadenosine (2-CADO; 100 pM–10 μM), R-N6-phenylisopropyladenosine (R-PIA; 10 pM–10 μM), S-PIA (100 pM–100 μM), cyclohexyladenosine (CHA; 10 pM–1 μM), and cyclopentyladenosine (CPA; 10 pM–1 μM). The A_2 receptor agonist, 2-[4-(2-carboxyethyl)phenylethylamino]-5-N-ethylcarboxamidoadenosine (CGS21680; 100 pM–10 μM), was also used. Nonspecific binding was determined with 100-μM adenosine.

Tissues were incubated at room temperature for 1 h and then terminated by vacuum filtration over GF/B filters on a cell harvester (Brandel, Gaithersburg, MD) with three washes of 4 ml cold 170 mM Tris buffer. All experiments were performed in duplicate. Radioactivity bound was quantified by immersion of filters in 10 ml Biosafe II scintillation fluid counted in a scintillation counter. Saturation curves were constructed and analyzed with GraphPad Prism (GraphPad Software Inc., San Diego, CA).

Statistics

Data are presented as mean ± SE or median ± quartiles, as indicated. Changes of adenosine concentration in CSF microdialysates after drug application compared with baseline were statistically analyzed using one-way repeated-measures analysis of variance followed by the Dunnett test. $P < 0.05$ was considered significant.

Results

Microdialysis

The median baseline concentration of two consecutive samples of adenosine in the dialysate was 0.007 μM [0.005 μM 25th, 0.014 μM 75th percentiles] in normal rats and 0.008 μM [0.007 μM, 0.015 μM] in spinal nerve–ligated rats (P = not significant between normal and nerve-ligated animals; fig. 1). Intrathecal injection of 20 μg adenosine led to an increase of the adenosine concentration in both normal and spinal nerve–ligated animals with a maximum 5 min after injection in normal animals (median: 2.97 μM [0.97 μM, 22.1 μM]) and 10 min in ligated animals (median: 0.68 μM [0.28 μM, 8.8 μM]). In the following 10 min, the concentration decreased, and after 20 min, the adenosine had totally disappeared in both groups. The measured concentrations at 5 and 10 min were significantly different from baseline in both groups, but there was no change in the time course of adenosine's disappearance between normal and spinal nerve–ligated animals.

Animal Study: Radioligand Binding

[^{3}H]-DPCPX bound to a single site, whether experiments were performed with or without adenosine deaminase (fig. 2). Maximum specific binding was 85% in the absence of adenosine deaminase and 95% in the presence of adenosine deaminase. In lumbar spinal cord, binding differed according to treatment (spinal nerve ligation *vs.* normal), and incubation conditions (adenosine deaminase present *vs.* absent). Thus, maximal binding (B_{max}) was increased 75–100% in lumbar spinal cord in animals after spinal nerve ligation compared with controls, without a change in binding affinity (fig. 2 and table 1; $P < 0.01$ between B_{max} in either group without and with adenosine deaminase). In contrast, lumbar spinal nerve ligation had no effect on total binding or binding affinity in dorsal quadrants of thoracic spinal cord, whether adenosine deaminase was added or not (table 2). Competition studies revealed a single site in spinal nerve–ligated animals, with inhibition constants K_i for A_1 ligands of 0.7 nM (CPA), 1.1 nM (CHA), 1.7 nM (R-PIA), 15 nM (2-CADO), and 39 nM (S-PIA), and K_i for the A_2 ligand CGS 21680 of 123 nM.

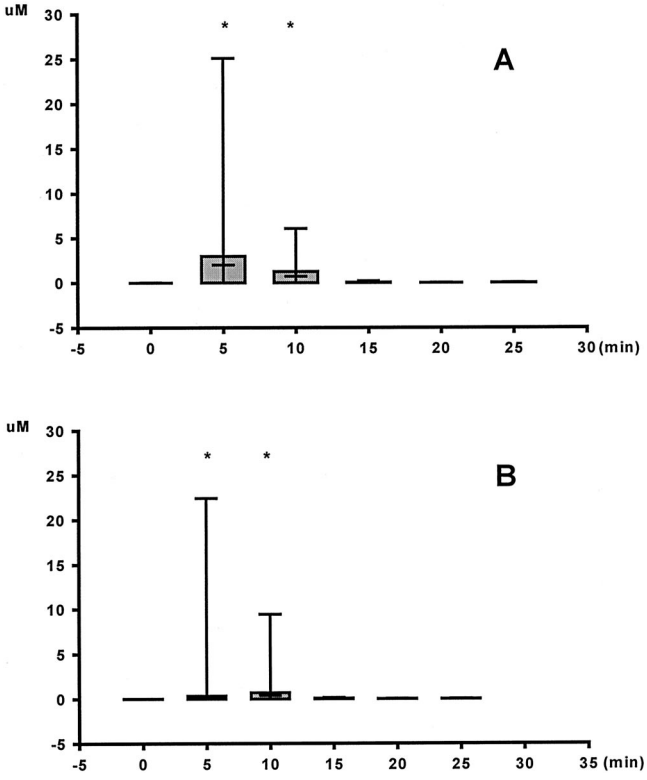

Fig. 1. Time course of adenosine concentration in artificial cerebrospinal fluid after intrathecal injection of 20 μg adenosine. Each bar represents the median ± 75th and 25th percentiles of eight animals. Baseline at time point 0 indicate the average of two consecutive 5-min samples. (*A*) Normal rats, (*B*) spinal nerve–ligated rats. *$P < 0.05$ compared with baseline.

Discussion

The current study describes three key aspects of mechanism of action of intrathecally administered adenosine for the treatment of pain: lack of pharmacokinetic explanation for the prolonged duration of action, lack of up-regulation of adenosine A_1 receptor number as an explanation for increased efficacy after spinal nerve ligation, and suggestion that there is a depletion of spinal cord adenosine after spinal nerve ligation. Our companion clinical article, although studying normal volunteers and acute sensitization by intradermal capsaicin injection rather than nerve injury, is in accordance with the current findings in showing a brief (< 4 h) residence time of adenosine in CSF but a prolonged (24 h) duration of action after intrathecal bolus.[17]

Intrathecal Space Pharmacokinetics

Sampling of CSF to determine pharmacokinetics of drugs in CSF after intrathecal administration, especially in small animals, such as rats, is problematic. Not only is the CSF volume small, but removal of fluid for analysis, even with concomitant replacement with an equivalent volume of artificial CSF, can alter drug kinetics and distribution in the spinal intrathecal space. This is suggested in humans by the dramatically different pharmacokinetics and dynamics observed between single intrathecal injection of neostigmine through a No. 27 Whitacre needle without CSF sampling compared with injection through a No. 22 catheter with sampling.[18] For these reasons, we chose to use microdialysis of the intrathecal space,[12] which does not alter drug distribution by sampling artifacts, although it does introduce problems of mechanical obstruction in the intrathecal compartment, variable efficiency of dialysis probe recovery, and limited time resolution.[19]

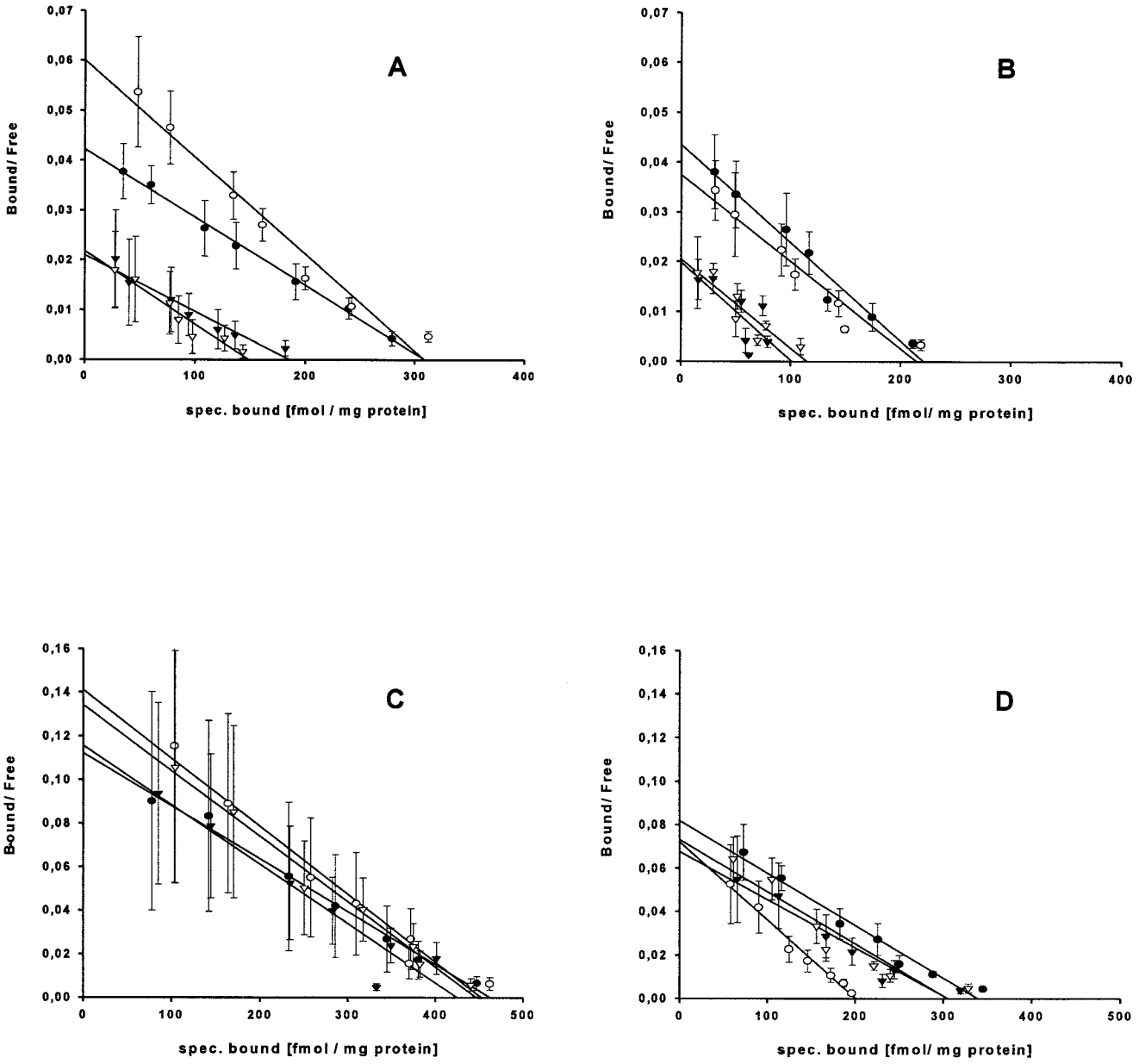

Fig. 2. (*A* and *C*) Scatchard plots of left and right dorsal horns of spinal nerve–ligated (SNL) and normal rats. Experiments in (*A*) were performed without adding adenosine deaminase to membrane preparations for total binding. (*B*) Data of experiments obtained with adenosine deaminase. Open circles: SNL—left lumbar dorsal; closed circles: SNL—right lumbar dorsal; open triangles: normal—left lumbar dorsal; closed triangles: normal—right lumbar dorsal. (*B* and *D*) Scatchard plots of ventral horns of spinal nerve–ligated and normal animals. Open circles: SNL—left lumbar ventral; closed circles: SNL—right lumbar ventral; open triangles: normal—left lumbar ventral; closed triangles: normal—right lumbar ventral. (*B*) Without adenosine deaminase, (*D*) with adenosine deaminase. Each symbol indicate the average ± SD of three experiments performed in duplicate with n = 9 or 10 animals in each experiment.

Despite the qualifications necessary in performing pharmacokinetics using microdialysis probes, the current study provides two clear observations regarding intrathecal adenosine disposition. First, there was an obvious discrepancy between the duration of action to reduce mechanical hypersensitivity in this model in recent studies from the same laboratory (12–24 h)[11,20] and the rapid disappearance of increased concentrations of adenosine in CSF within 30 min in the current study. Of course, this does not exclude a depot of adenosine in other neuraxial compartments, such as epidural fat or spinal cord tissues, but clearly shows that adenosine does not produce long-lasting effects by residing in CSF. Second, there is no difference in the resting concentrations of adenosine in CSF between normal and spinal nerve–ligated animals, nor is there a difference in the disappearance from CSF between these groups after exogenous bolus of adenosine in CSF.

The causes for the long pharmacodynamic effect of intrathecal adenosine in rats with peripheral nerve injury

Table 1. Adenosine Receptor Binding in Lumbar Spinal Cord

Tissue	B_{max} (fmol/mg protein)	K_d (pM)	B_{max} (fmol/mg protein)	K_d (pM)
	Without Adenosine Deaminase		With Adenosine Deaminase	
SNL				
Right lumbar dorsal horn	313.9 ± 16.20*	613.4 ± 92.69	470.9 ± 41.02	401.4 ± 118.7
Left lumbar dorsal horn	328.1 ± 20.28*	518.0 ± 98.13	466.1 ± 31.76	310.7 ± 77.69
Right lumbar ventral horn	224.7 ± 19.95*	498.9 ± 136.9	350.8 ± 20.55	355.4 ± 73.36
Left lumbar ventral horn	223.2 ± 15.18*	546.8 ± 112.3	204.6 ± 9.46*†	222.3 ± 41.77
Normal				
Right lumbar dorsal horn	188.2 ± 21.71	669.0 ± 233.6	405.1 ± 28.26	267.3 ± 74.02
Left lumbar dorsal horn	140.1 ± 17.90	380.8 ± 175.4	464.9 ± 44.45	316.3 ± 111.5
Right lumbar ventral horn	73.15 ± 11.45	172.7 ± 122.8	317.4 ± 24.86	372.4 ± 102.4
Left lumbar ventral horn	103.2 ± 15.95	556.6 ± 265.1	336.1 ± 31.56	532.9 ± 158.0

* Significant difference from the equivalent tissue in normal animals. † Significant difference from the contralateral side.
SNL = spinal nerve ligation.

are unknown. Possibilities include repletion of depleted adenosine stores in spinal cord tissue itself (see A_1 Adenosine Receptor Binding section, third paragraph); prolonged activation of A_1 receptors, leading to decreased activity of adenosine kinase, thereby decreasing adenosine's uptake[21,22]; or transient blockade of the sensitization process, which could take several hours to recover.

A_1 Adenosine Receptor Binding

The radioligand binding studies in the presence of adenosine deaminase to remove endogenous adenosine remaining in the membrane homogenates show no difference in either affinity or quantity of A_1 adenosine receptors in dorsal spinal cord after spinal nerve ligation. Others have speculated that the enhanced antinociceptive action of adenosine kinase inhibitors, like adenosine itself, in rats after spinal nerve ligation, might reflect increased A_1 adenosine receptors,[1] which is clearly not supported by the current results. It is conceivable that receptor density in a small area of the spinal cord could change without being reflected in this homogenized tissue assay. We believe it is unlikely that we missed such a change because A_1 adenosine receptors are concentrated in superficial laminae of the spinal cord, and we examined dorsal spinal cord tissue, which was separated ipsilateral and contralateral to nerve injury, with no evidence of a difference between them.

However, there was a regional difference in adenosine receptor number, localized to the lumbar area—the site of nerve injury—when adenosine deaminase was not added to the membrane preparation. Endogenous adenosine, probably from both extracellular and intracellular sources, partitions into membranes during this preparation, and for this reason, adenosine deaminase is added to remove this endogenous competing ligand to determine receptor number in radioligand binding or G-protein activation experiments.[23] Increased B_{max} in the lumbar region of nerve-injured compared with normal rats in the absence of adenosine deaminase in the current study is most consistent with a reduction in endogenous competing adenosine in the nerve-injured animals, allowing more binding sites available to the radioligand.

Adenosine depletion after spinal nerve ligation is supported by several observations: alleviation of allodynia by intrathecal injection of an adenosine reuptake inhib-

Table 2. Adenosine Receptor Binding in Thoracic Spinal Cord

Tissue	B_{max} (fmol/mg protein)	K_d (pM)	B_{max} (fmol/mg protein)	K_d (pM)
	Without Adenosine Deaminase		With Adenosine Deaminase	
SNL				
Right thoracal dorsal horn	230.0 ± 25.60	762.8 ± 237.5	326.2 ± 30.53	386.4 ± 125.8
Left thoracal dorsal horn	175.1 ± 11.28	520.9 ± 103.7	303.3 ± 33.30	262.0 ± 112.7
Right thoracal ventral horn	150.8 ± 23.04*	646.6 ± 300.9	179.3 ± 11.60	267.0 ± 64.60
Left thoracal ventral horn	174.9 ± 35.03*	1434 ± 702.6	182.3 ± 8.11	212.8 ± 37.83
Normal				
Right thoracal dorsal horn	186.2 ± 17.10	785.9 ± 198.3	321.0 ± 20.11	391.5 ± 83.57
Left thoracal dorsal horn	155.2 ± 8.20	442.2 ± 77.54	298.9 ± 17.81	233.7 ± 55.59
Right thoracal ventral horn	75.42 ± 7.26	397.1 ± 132.3	163.7 ± 15.07	271.8 ± 93.14
Left thoracal ventral horn	94.51 ± 15.19	884.1 ± 386.7	201.4 ± 17.31	315.8 ± 98.66

* Significant difference from the equivalent tissue in normal animals. † Significant difference from the contralateral side.
SNL = spinal nerve ligation.

itor,[11] reduced adenosine release by morphine in spinal cord from animals after spinal nerve ligation (preliminary observations), and reductions in CSF concentrations of adenosine in patients with chronic pain.[24] Measuring resting intracellular adenosine concentrations or the half-life of increasing intracellular adenosine concentrations by bathing neurons with adenosine, such as would occur after intrathecal injection, would be difficult to study, given the rapid cycling of this purine nucleoside in transcription and energy processes. However, we propose that the selectivity of intrathecal adenosine to hypersensitivity states and its prolonged duration of action could share a common explanation: increased intracellular adenosine concentrations after intrathecal adenosine injection and restoration of depleted adenosine stores.

In summary, intrathecal adenosine disappears rapidly from the intrathecal CSF space in rats but has a prolonged duration of action, indicating that long residence time in CSF is not the cause of its prolonged effect. Radioligand binding studies are not consistent with upregulation of spinal adenosine receptors as a cause for increased activity of intrathecal adenosine in hypersensitivity states. Rather, they support an association between depletion of spinal cord adenosine and allodynia in this model. We propose that intrathecal adenosine's effect is amplified in hypersensitivity conditions and is long lasting because it primarily acts to increase intracellular concentrations of adenosine in intrinsic spinal cord neurons.

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